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Isolation, Identification, and Expression of Microbial Cellulases from the Gut of *Odontotermes formosanus*

Jiwei Duan[1†], Jun Liu[2†], Xueling Ma[3], Yue Zhang[1], Xiaohua Wang[1*], and Kai Zhao[1*]

[1]*Key Laboratory of Microbiology, School of Life Science, Heilongjiang University, Harbin 150080, P.R. China*
[2]*College of Life Science Engineering, Qiqihar University, Qiqihar 161005, P.R. China*
[3]*Department of Neurosurgery, the Fourth Affiliated Hospital of Harbin Medical University, Harbin 150001, P.R. China*

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*Corresponding authors
K.Z.
Phone: +86-451-86608586;
Fax: +86-451-86609016;
E-mail: zybin395@126.com
X.W.
Phone: +86-451-86608586;
Fax: +86-451-86609016;
E-mail: 617427207@qq.com

†These authors contributed equally to this work.

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Termites are destructive to agriculture, forestry, and buildings, but they can also promote agro-ecosystem balance through the degradation of lignocellulose. Termite-triggered cellulose digestion may be clarified through microbial metabolism of cellulose products. In the present study, we characterized the activities of cellulase and its three components synthesized by the cellulase-producing fungal strain HDZK-BYTF620 isolated from the gut of *Odontotermes formosanus*. The protein components of cellulases were synthesized by strain HDZK-BYTF620, which were isolated and characterized using polyacrylamide gel electrophoresis, and the expression of the cellulases was studied at the proteome level.

Keywords: Termites, endophyte, cellulase decomposing strain, proteomics, biocontrol

Introduction

Termites cause considerable damage to natural and man-made structures as well as crops, resulting in large economic losses every year. Because of the microbial biodiversity in termite guts, they are the best decomposers of lignocellulose-containing materials in nature [1]. Lower termites harbor eukaryotes and prokaryotes, with different distributions among the gut compartments [2]. The expression of genes involved in caste differentiation [3] and cellulose digestion has received the most attention in termite genomics [4–6] and proteomics [7]. These gut microbes are especially important for wood-eating termites. They provide efficient digestion of wood [8], particularly the cellulose components.

Microbial cellulases from termite guts have been widely studied [9–11]. Termites have two cellulose-digesting systems; one consists of endogenous cellulases, and the other is composed of cellulases from symbiotic bacteria and protozoa [12]. Both systems, along with the morphology of termites and the great number of microbes present in the gut, contribute to efficient digestion of lignocellulose [13]. Termites accomplish cellulose digestion through the synergistic effect of three types of cellulases, including endoglucanases, exoglucanases, and β-glucosidases [14]. Termites and fungi have established symbiotic relationships [15].

The subterranean fungus-growing termite *Odontotermes formosanus* is a serious pest of trees and dams in China. Aside from classical chemical control technologies, efforts have been made to develop biological control methods, including work to understand the possibilities of deploying an insect pathogenic fungus, *Metarhizium anisopliae*, to control *O. formosanus* populations, showing that the system has a relatively low oxygen level, neutral gut pH, and

higher redox potentials and higher hydrogen accumulation than other termite species [16, 17]. The authors interpreted their findings in terms of a specialized gut system for fungus-growing termites. Most recently, Huang *et al*. [18] published a transcriptome derived from the heads of *O. formosanus*, providing useful sequence information for future studies. However, this information on head-specific gene expression does not help in understanding its cellulose digestion. Here, we provided biological background on this destructive pest, with our analysis of cellulases expressed by the fungal strain HDZK-BYTF620 isolated from the gut of *O. formosanus*. The termite's abdomen was disinfected by 75% alcohol and transferred into a mortar containing sterile phosphate buffer (PBS, pH 7.4). The termite's abdomen was ground into a homogenate, and using the gradient dilution plate coating method, a purified single colony was obtained by standard plate-streaking technique. At the same time, after disinfection of the termite's abdomen on the plate, this plate and the control plate were together cultivated to test whether the surface disinfection of termites was complete. After the isolation and purification, the bacterial strain was grown in the culture medium with cellulose as the sole carbon source. A transparent circle appeared around the colony after 72-h culture, showing that the strain could degrade cellulose. In addition, strain HDZK-BYTF620 was identified as *Aureobasidium pullulans* through morphological observation as well as molecular and biological identification.

Materials and Methods

Insects and Strains for Bioassay

The termite species, *O. formosanus*, was collected from Logia Hill of Wuhan in China. Termites were maintained in an incubator at 26.5°C, and 80% RH was supplied with water and newspaper.

The fungal strain HDZK-BYTF620 with cellulose-decomposing activity, isolated from the gut of *O. formosanus*, was stored in the Key Laboratory of Microbiology, School of Life Science, Heilongjiang University, China.

Fungal Culture

Strain HDZK-BYTF620 was activated on potato dextrose agar (PDA) slant medium consisting of 200 g/l potato, 10 g/l glucose, and 15 g/l agar at 28°C for 72 h. In order to evaluate the cellulase production capacity, mycelia of strain HDZK-BYTF620 were inoculated into medium plates and cultured at 28°C for 5 days. Each plate was stained with Congo red for 60 min and then washed with 1.0 M NaCl until the washing solution became clear.

The mycelia from the PDA slant medium were transferred to seed culture medium consisting of 200 g/l peeled and cut potato and 10 g/l glucose, and then grown at 28°C in a rotary shaker at 180 rpm for 3 days. The culture was inoculated into a fermentation medium (21.88 g/l sodium carboxymethyl cellulose (CMC-Na), 0.77 g/l $CaCl_2$, 9 g/l NaCl, 0.41 g/l $MgSO_4$, 0.5 g/l KH_2PO_4, 1 g/l $(NH_4)_2SO_4$, pH 6.0) using CMC-Na as the only carbon source. After inoculation, the liquid was incubated at 28°C, 180 rpm for 7 days.

Isolation of Cellulase Components

SDS-PAGE gels were used to isolate the cellulases after the extract from termites was purified. The gels were stained with Coomassie Brilliant Blue R-250 dye for 1 h, washed three times with distilled water, and then destained three times with Coomassie Blue detaining solution until the bands became clearly visible. The gels were scanned, and images were taken and saved.

The appropriate protein fraction within the gel was determined using renaturation electrophoresis, and the type of cellulase was then assessed. CMC-Na was added into the substrate during preparation of the resolving gel and stacking gel, and the samples were not boiled before electrophoresis. After renaturation electrophoresis, 8% resolving gel (4.6 ml of 1% CMC-Na, 2.7 ml of 30% acrylamide, 2.5 ml of 1.5 M Tris-HCl, 0.1 ml of 10% SDS, 0.1 ml of 10% AP, and 0.006 ml TEMED) and 5% stacking gel (3.4 ml of 1% CMC-Na, 0.83 ml of 30% acrylamide, 0.63 ml of 1.5 M Tris-HCl, 0.05 ml of 10% SDS, 0.05 ml of 10% AP, and 0.005 ml TEMED) were conventionally stained for cellulase activity. The ratio of sample to renaturation electrophoresis buffer was 4:1. The mixture was added into a 1 ml centrifuge tube and mixed at room temperature for 5 min, and the sample quantity of each pore was 15 µl. The procedure of conventional staining was the same as for SDS-PAGE. The starting current was 10 mA, and then it was adjusted to 15 mA. The gels were prestained to record enzyme activity. An SDS washing liquid was used to cover the surface of the gel. The gel was washed for 30 min on an oscillating plate. After 15 min, the SDS washing liquid was removed, and deionized water was used to wash the gel for three times (5 min/wash) [19, 20]. After staining with Congo red for 1 h, the gels were decolorized with NaCl solution until the elution liquid became clear. Images of the gels were captured and stored.

Identification of Cellulase

The gel bands containing exoglucanase and β-glucosidase were excised. Avicel and 1% salicin solutions were used as substrates for exoglucanase and β-glucosidase, respectively.

Each gel band was cut into granular pieces of 1–2 mm^3, which were then transferred into 1 ml Eppendorf tubes. Approximately 100 µl of detaining solution was added into the tube, and then discarded. Subsequently, 80–100 µl of HPLC-grade acetonitrile was added, and the tubes were incubated for 30 min. The gel particles shrank to white lumps, which were briefly dried at room temperature. A trypsin solution (50 mM NH_4HCO_3 containing 6 mg/ml trypsin) was added to the gel pieces, followed by incubation at 37°C for 10 h. The peptides were extracted with 1%

formic acid and 2% acetonitrile for 1 h, followed by extraction with 60% acetonitrile for 1 h. The two extracts were combined and lyophilized. The pellet was dissolved in 1% formic acid or 2% acetonitrile, and then it was desalted using the Zip-Tip C18 micro column (EMDMillipore, USA) according to the manufacturer's instructions. Briefly, Millipore Zip Tip C18 columns were activated using a neutral solution of 50% ACN and balanced with 0.1% formic acid solution. The enzyme digest was dissolved using 2 µl of 0.1% formic acid solution, and 0.1% formic acid was used to wash out the salt. Thereafter, the peptide samples were sent to the Proteomics and Mass Spectrometry Core at the University of Florida (USA) for analysis with HPLC-MS/MS [21].

Glucose Standard Curve

Graduated cylinders (25 ml) with stoppers were numbered 0, 1, 2, 3, 4, 5, and 6, with No. 1 to No. 6 tubes as replicates. Glucose standard solution, distilled water, and dinitrosalicylic acid (DNS) reagent were added to prepare the reaction liquid with various glucose contents. The optical density of tubes 1–6 was determined at a wavelength of 540 nm using tube 0 as a blank to create a standard curve.

Cellulase Activities

The model substrates were used to test total cellulase (Xinhua filter paper; Fushun Civil Administration Filter Paper Factory, China), endoglucanase, (CMC; Tianjin Guangfu Fine Chemical Research Institute, China), exoglucanase (Avicel), and β-glucosidase (Avicel and salicin from Tianjin Guangfu Fine Chemical Research Institute) activities. After adding acetate buffer, the boiled control and replicate samples were incubated at 50°C in a water bath. After 2 min incubation, the DNS reagent and NaOH solution were added, and OD_{540nm} values were determined accordingly.

2D Gel Electrophoresis of Cellulase Components

Fermentation medium was extracted at 24, 48, 72, 96, and 120 h according to the requirements of the 2D gel electrophoresis. Before isoelectric focusing, the prepared enzyme sample was removed from –20°C and dissolved in hydration solution. Briefly, 450 µl of sample was added into the focusing groove, and the side of the IPG gel strip was put into the focusing groove. After isoelectric focusing, the gel strips in the equilibrium solution 1 and equilibrium solution 2 were balanced for 15 min. After the equilibrium, the gel strips were transferred to 8% SDS-PAGE gel. Subsequently, silver nitrate staining was performed, followed by image scanning, analysis, and preservation.

Statistical Analysis

The Scheffe multiple comparison procedure from the SAS statistical program was employed to evaluate differences in nitrogenase activity. Results with $p < 0.05$ were considered statistically significant. All the experiments were repeated three times and each measured in triplicate. All results are expressed as the mean ± standard deviation.

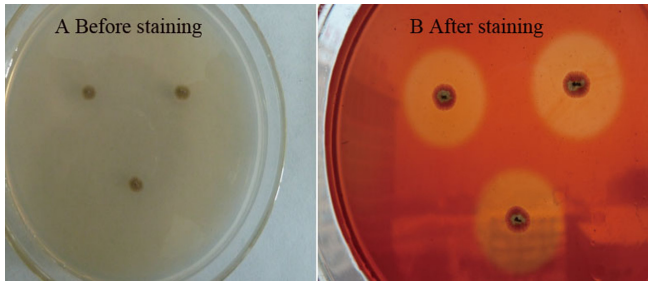

Fig. 1. Validation of cellulase-producing capacity of strain HDZK-BYTF620.
(**A**) Before Congo red staining and bleaching; (**B**) After Congo red staining and bleaching. For cellulase producing capacity analysis, transparent zones can be seen in **B** but not in **A**.

Results

Validation of Cellulase Capacity

After staining and destaining of Congo red, areas around the colonies became transparent, verifying the expression of cellulase in strain HDZK-BYTF620 (Fig. 1).

Determination of Cellulase

Fig. 2A shows that clear transparent bands appeared on the gel after staining and destaining of Congo red, indicating the location of endoglucanase.

Figs. 2A and 2B show the results of two staining methods, conventional gel staining and staining for enzyme activity. As shown in Fig. 2C, bands 2 and 3 corresponded to the obvious transparent band 9 on the refolding gel electrophoresis. The endoglucanase component was initially identified in bands 2 and 3. The corresponding position of band 5 had a more obscure transparent band 10, indicating that it might contain a relatively small endoglucanase.

PAGE Gels

Our data showed that except for the endoglucanase activity that peaked during 72 h, the activities of other enzymes barely changed. Therefore, we selected a fermentation time of 72 h to conduct the electrophoresis. In Fig. 2B, bands 1–4 were visible, with corresponding sizes of 86, 65, 60, and 50 kD, and the bands 5–7 were vague. This result suggested that subunits 1–4 had relatively high expression, whereas subunits 5–7 had relatively low expression levels.

Isolation of Cellulase Components

The gel bands in refolding electrophoresis showed exoglucanase and β-glucosidase activities. Fig. 3 shows the presence of exoglucanase activity in bands 1–3 and β-

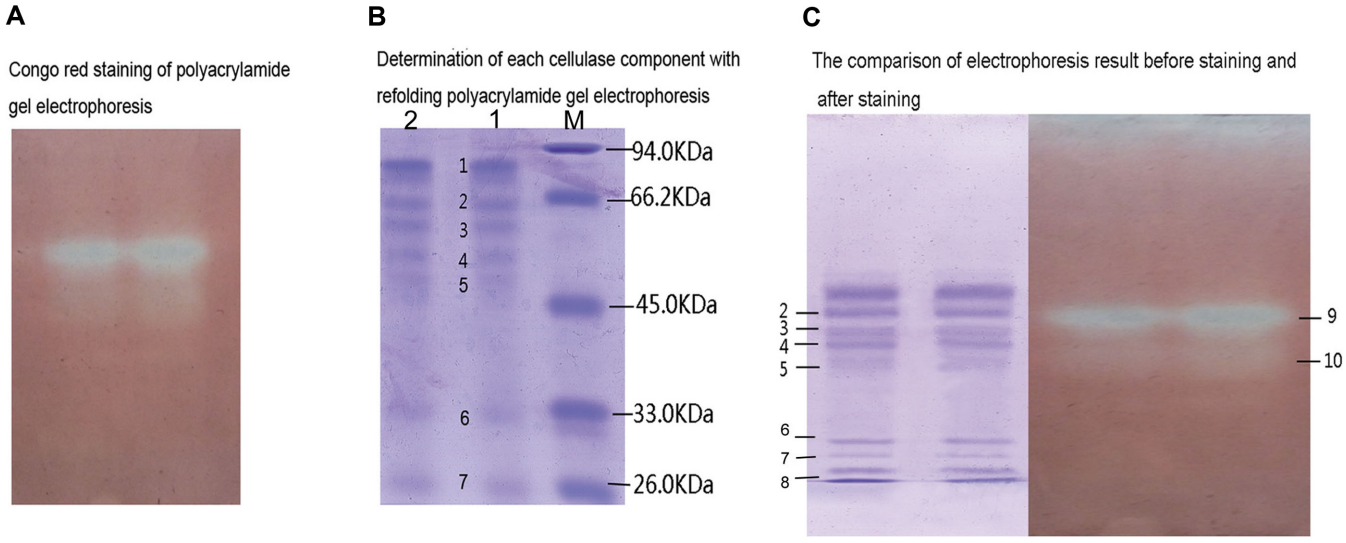

Fig. 2. Identification of eight cellulase components in the gut of termite through SDS-PAGE and refolding-PAGE.
(**A**) Congo red staining of polyacrylamide gel electrophoresis. (**B**) Determination of cellulase component with refolding PAGE. Lanes 1 and 2 are fermented cellulase protein. (**C**) Comparison of electrophoresis before staining and after staining. Bands 1–3 represent exoglucanase activities; Bands 5–6 represent β-glucosidase activity; Bands 4, 7, and 8 represent no cellulase activity.

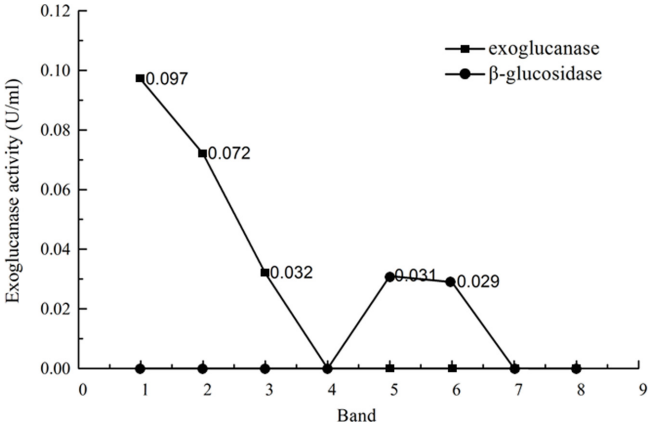

Fig. 3. Determination of the cellulase activity of each component identified in refolding PAGE.

glucosidase activity in bands 5 and 6.

Identification of Cellulase

Strain HDZK-BYTF620 was cultured for 72 h, and then the culture solution was stained and destained for SDS-PAGE. According to the method of cellulase identification, the decolorization and dehydration of gel particles were performed for HPLC-MS/MS analysis. As seen in Table 1, points 1–3 matched exoglucanase, point 3 matched endoglucanase, and point 4 had no cellulase component. In addition, Table 1 shows that point 1 and exoglucanase 1 were highly matched; therefore point 1 might be identified as exoglucanase precursor 1. The number of matched peptides and the sequence coverage of exoglucanase 2 were higher than those of exoglucanase 1 in point 2, indicating that point 2 was mainly exoglucanase precursor 2, but there was also an exoglucanase precursor 1. Point 3 matched endoglucanase 4, whereas there was a lower matching between exoglucanase 2 and point 2. There was an overlap between the endoglucanase precursor and exoglucanase precursor at a certain degree, which were not completely separated during electrophoresis. Points 5 and 6 matched endo-1,4-β-xylanase A and endo-1,4-β-xylanase, respectively. However, the pI of point 6 matching endo-1, 4-β-xylanase was 6.33, which was slightly alkaline. The other component was slightly acidic, and the optimum pH was 6.0 to produce cellulose by the strain HDZK-BYTF620. There were significant differences between point 7 and point 8 in terms of experimental and theoretical molecular weight, but the number of matched peptides and the sequence coverage were quite similar.

Endoglucanase Activity

Fig. 4A shows the endoglucanase activity recorded over the 120-h fermentation period. The activity was gradually increased to a maximum of 1.3 ± 0.2 IU/ml at 72 h, and there was a significant difference from 24 to 120 h ($p < 0.05$). After that, the endoglucanase activity was decreased to about 1.0 IU/mL for the remaining incubation period.

Table 1. Matched results between the amino acid sequences and database information.

Protein point	Number	Expression proteins (subunits)	Comparison score	Matching peptide	Sequence coverage (%)	Molecular weight (D)	Determined MW (D)	pI	Species
1	GUX1_PENJA	Exoglucanase 1	151	3	6	56,808	86,000	5.38	*Penicillium janthinellum* (*Penicillium vitale*)
2	GUX2_TRIRE	Exoglucanase 2	304	5	12	49,622	65,000	5.11	*Trichoderma reesei* (*Hypocrea jecorina*)
	GUX1_PENJA	Exoglucanase 1	142	2	6	56,808	65,000	5.38	*Penicillium janthinellum* (*Penicillium vitale*)
3	GUX2_TRIRE	Exoglucanase 2	48	1	2	49,622	60,000	5.11	*Trichoderma reesei* (*Hypocrea jecorina*)
	GUN4_TRIRE	Endoglucanase 4	37	1	2	35,488	60,000	5.29	*Trichoderma reesei* (*Hypocrea jecorina*)
4	-	-	-	-	-	-	-	-	-
5	XYNA_ASPKA	Endo-1,4-β-xylanase A	35	2	4	35,416	47,000	5.91	*Aspergillus kawachi* (*Aspergillus awamori* var. *kawachi*)
6	XYNA_ASPAC	Endo-1,4-β-xylanase	62	3	6	35,309	36,000	6.33	*Aspergillus aculeatus*
7	GUX1_PENJA	Exoglucanase 1	74	1	2	56,808	28,000	5.38	*Penicillium janthinellum* (*Penicillium vitale*)
8	GUX1_PENJA	Exoglucanase 1	83	1	2	56,808	28,000	5.38	*Penicillium janthinellum* (*Penicillium vitale*)

Exoglucanase Activity

The exoglucanase activity was increased to about 2.5 IU/ml, and it remained stable throughout the 120-h incubation period (Fig. 4B).

β-Glucosidase Activity

The β-glucosidase activity was increased to about 1.2 IU/ml over the first 48 h, and then it remained relatively stable throughout the incubation period (Fig. 4C).

Determination of Total Cellulase Activity

Filter paper has a relatively loose structure with many non-reducing ends, which can be degraded by both endoglucanase and exoglucanase and then changed to glucose by β-glucosidase. Fig. 4D shows that the cellulase activity was stable at about 2.3−2.8 IU/ml over the incubation period.

2D Gel Electrophoresis of Cellulase Components

Fig. 5 shows that five distinct proteins (subunits) were expressed during the fermentation. The results indicated that the five subunits of three enzyme components were involved in the degradation of cellulose in the fermentation culture. It also showed that the degradation of cellulose was caused by a multi-enzyme synergistic action.

Discussion

In order to verify whether the fungal strain HDZK-BYTF620 had cellulose-decomposing activity, we determined the endoglucanase activity using the substrate CMC-Na. Exoglucanase is highly specific for the cellulose chains, and endoglucanase has low specificity for carboxymethyl cellulose degradation. The result in Fig. 1 suggested that the strain was able to decompose cellulose.

In the present study, we described the protein expression profile in the strain isolated from the termite gut. Only a few studies have addressed the underlying proteomic processes that drive reproduction in termites [22-26]. Before the rise in proteomics, the studies of cellulase mainly focus on the gene level, and EG I (endoglucanase from fungi, referred to as EG), EG III, CBH I (exoglucanase from fungi, referred to as CBH), CBH II, CBH III, CBH IV, and BG (β-glucosidase, referred to as BG) genes have been cloned and expressed [27]. With the application of proteomics technology, the separation and identification of cellulase components have been completed.

Cellulase is a multicomponent enzyme. In order to

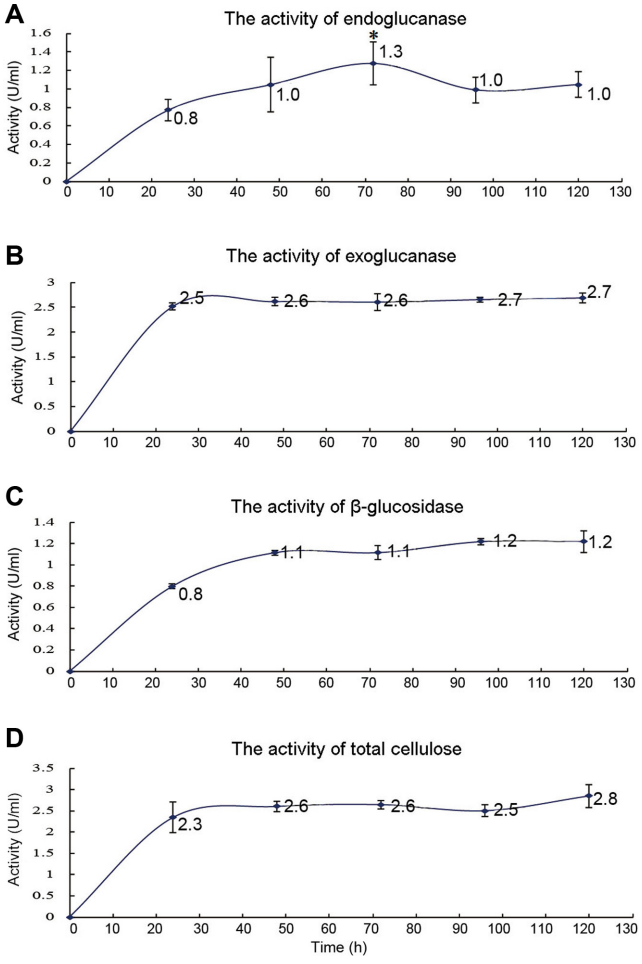

Fig. 4. Profiles of cellulase activity in culture broth during the cultivation of strain HDZK-BYTF620.
(**A**) Activity of endoglucanase; (**B**) Activity of exoglucanase; (**C**) Activity of β-glucosidase; (**D**) Activity of total cellulase. * indicates a significant difference at $p < 0.05$ or 0.01.

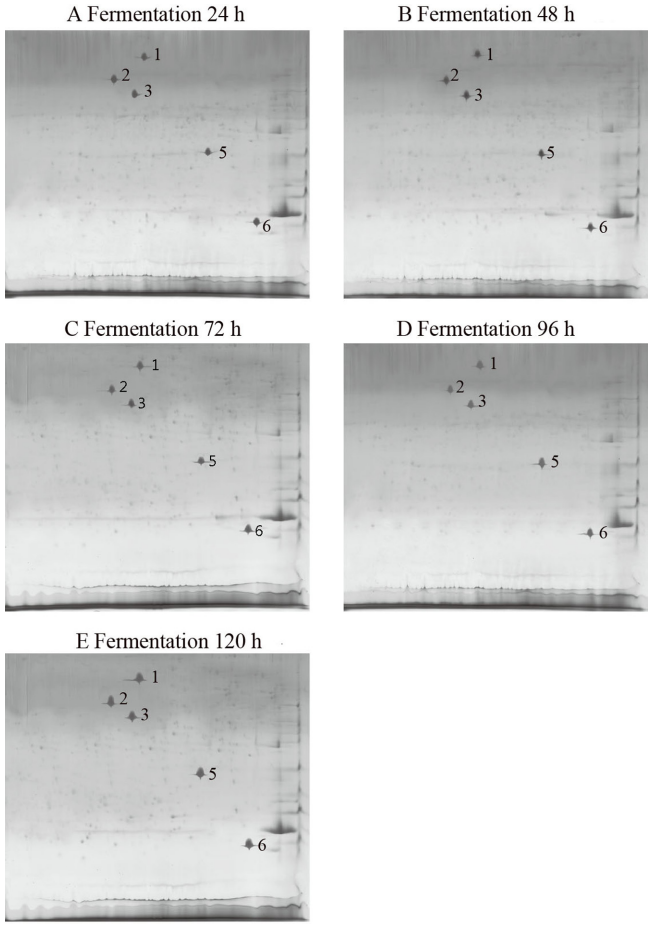

Fig. 5. Determination of cellulase expression from strain HDZK-BYTF620 in different fermentation periods by electrophoresis analysis.
(**A**) Fermentation of 24 h; (**B**) Fermentation of 48 h; (**C**) Fermentation of 72 h; (**D**) Fermentation of 96 h; (**E**) Fermentation of 120 h.

separate and identify the obtained cellulase components, we used a common technique of the protein separation. Moreover, renaturation electrophoresis was carried out to assess the effects of cellulase on specific substrates, providing a preliminary understanding of the component before mass spectrometric analysis. In renaturation electrophoresis, samples are not completely denatured before electrophoresis, and the enzyme is refolded in the gel-substrate reaction. After staining of biological activity, enzymes on the corresponding sites can be observed.

The number of bands in Fig. 2 was inconsistent, probably because the process of complex electrophoresis exerted less destruction on samples compared with the conventional gel electrophoresis. The electrophoresis sample buffer without β-mercaptoethanol maintained the disulfide bonds of protein, and the mixture was not boiled, leading to less damaged protein structure of enzymes. Some researchers have pointed out that boiling or the mixture of sample and sample buffer has no effect on the electrophoresis, indicating that the sample in conjunction with the sample buffer without boiling can also degenerate [28]. After mass analysis, we identified three types of cellulase components, including a total of five subunits from strain HDZK-BYTF620. Point 3, containing presumably endoglucanase 4 and exoglucanase 2, showed a degree of polymerization. Point 6 of the identified endo-1,4-beta-xylanase had a higher pI. Points 3 and 6 still require further study.

For expression assays of the cellulase components, the recombinant enzyme combinations were set up in a number

of reactions containing each enzyme at different molar ratios, where the total protein concentration was kept constant [29, 30]. In order to determine the cellulase activity, a proteomics analysis was performed, and a set of candidate enzymes identified during the course of the study were examined. The expression of subunits generally reached a maximum at 72 h, and the level of enzyme activity peaked after 72 h, which was consistent with a previous study [31]. The cellulase activity was affected by the cellulose enzyme of each component of the subunits.

In order to analyze the cellulase expression of strain HDZK-BYTF620 in different fermentation periods, the cellulose was separated by 2D electrophoresis after fermentation for 24, 48, 72, 96, and 120 h. Fig. 5 shows the results. According to the molecular weight and pI of target proteins related to cellulase, we found that five major cellulase components (exoglucanase 1, exoglucanase 2, endoglucanase 4, endo-1,4-beta-xylanase A, and endo-1,4-beta-xylanase) identified from SDS-PAGE also appeared on 2D gel. There were five distinct proteins (subunits) in different fermentation periods, indicating that these five subunits were likely to be involved in cellulose degradation. The extent of cellulose degradation was likely controlled by the distribution of these cellulase components. Any component at low activity level may restrict the ability of cellulases to degrade cellulose [32], so the strain HDZK-BYTF620 has more comprehensive cellulase components to effectively degrade cellulose.

Cellulases are expressed by a wide spectrum of microorganisms in nature. The cellulases from bacteria and fungi are described and presented as aggregated structures attached to the cells. These extracellular and large enzyme aggregates are known as cellulosomes. Cellulases from fungi are commercially important, as these enzymes are secreted outside the cells and are robust. Isolation of cellulase-producing microbes from nature is one of the important ways to get novel cellulases. Therefore, our study promoted the cellulase production and also provided an approach of its production. The results showed that strain HDZK-BYTF620 was able to produce cellulase, and three components were synthesized by this cellulase-producing fungal strain isolated from the gut of *O. formosanus*.

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